

## Intracerebral Grafts of Neuronal Cell Suspensions

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### I. Introduction

As reviewed elsewhere in this volume, various methodological approaches have been developed for the grafting of neural tissue to the mammalian CNS. However, in adult recipients, grafting intraparenchymally to deep brain sites has offered special problems. Thus, insertion of solid pieces of fetal CNS tissue into the brain parenchyma, which works excellently in neonatal recipient rats (see Das, this volume), has been found to yield overall poor survival of the grafts in adults (Stenevi et al., 1976). It was this limitation that prompted the trials in our laboratory in 1979–1980 with injections of dissociated CNS cell suspensions (Björklund et al., 1980; Schmidt et al., 1981). Studies in vitro had already shown that embryonic CNS cells can survive an enzymatic dissociation procedure and retain many of their characteristic properties (see Fischbach and Nelson, 1977). The use of dissociated neuronal cell suspensions for transplantation has since demonstrated that the embryonic neurons implanted in this way can survive, proliferate, grow and express transmitter metabolism and function in several experimental models (see Table I and, e.g., Dunnett et al., Isacson et al. and Gage et al., this volume).

The procedure of cell suspension grafting involves three major steps, as summarized in Fig. 1. Firstly, dissection and collection of embryonic CNS tissue. Secondly, a dissociation step, which may or may not include incubation of the tissue pieces in trypsin, followed by washing and mechanical dis-

sociation of the tissue by pipetting. The third step is the stereotaxic injection of the cell suspension into the host brain.

TABLE I

TYPES OF DONOR TISSUE SUCCESSFULLY GRAFTED WITH THE SUSPENSION TECHNIQUE TO DIFFERENT TARGETS

Donor tissue (embryonic day)	Host target
Cerebellum (15) <sup>1</sup>	Cortex
Hippocampal formation (15-17) <sup>2</sup>	Cortex, hippocampus
Locus coeruleus (13-15) <sup>3</sup>	Hippocampus, spinal cord
Raphe nuclei (13-15) <sup>4</sup>	Hippocampus, spinal cord, caudate putamen
Septal-diagonal band (14-16) <sup>5,6</sup>	Hippocampus, cortex
Striatal eminence (14-15) <sup>7</sup>	Caudate putamen, substantia nigra
Substantia nigra (13-15) <sup>8</sup>	Caudate putamen, substantia nigra, nuc.
(Ventral mesencephalon)	accumbens, PF-cortex, amygdala, lateral hypothalamus, spinal cord

Suspensions from most of the regions listed above have been seen to survive also when accidentally, e.g. by retrograde flow, grafted to the lateral ventricles or neocortex.

References for the Donor tissue column are: <sup>1</sup> Schmidt et al., 1983; <sup>2</sup> Dunnett et al., unpublished; <sup>3</sup> Björklund et al., 1983; <sup>4</sup> Foster et al., this volume; <sup>5</sup> Björklund et al., 1983b; <sup>6</sup> Dunnett et al., 1985; <sup>7</sup> Isacson et al. this volume and unpublished; <sup>8</sup> Dunnett et al., this volume.

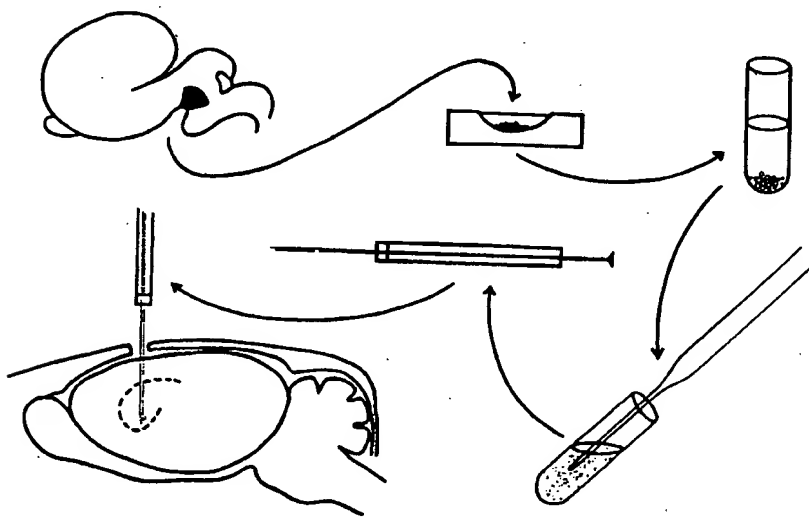


Figure 1. The principal steps: dissection, dissociation and injection, in the suspension grafting procedure (described in detail in text).

## II. Standard Procedure

### II.1. Preparation

All instruments and glassware are sterilized either by heat or in an autoclave. A basic medium consisting of 25–50 ml of 0.6% D-glucose in sterile 0.9% saline is prepared. About 3–7 ml of the basic saline-glucose medium is used to prepare a 0.1% trypsin (Sigma crude type II) solution (alternatively purified trypsin, Sigma type III, can be used at a concentration of 0.025%; Schmidt et al., 1983). The pregnant female rat is staged by palpation (see Seiger, this volume) and anesthetized with an intramuscular injection of a combination of ketamine (Ketalar, Parke-Davis; 50 mg/kg) and xylazine (Rompun, Hoechst; 10 mg/kg). The abdominal wall of the pregnant rat is washed with chlorohexidine before being cut open.

### II.2. Dissection

The pregnant rat is kept anesthetized until all the fetuses have been dissected. The fetuses are removed in groups of two to three at a time, beginning at the distal end of one uterine horn. The opened end of the uterine horn and the abdominal wall are kept closed by arterial clamps during the dissection.

The dissection procedure is not performed under sterile conditions, but care is taken to minimize potential bacterial contamination by handling the fetuses with sterilized instruments and glassware throughout. Different sets of instruments are used to open the mother, to remove the fetuses from the uterus, and to dissect the fetuses. The fetuses are placed on sterile glass microscope slides and the uterine wall and placenta are cut away revealing the embryo in its amniotic sac. The amniotic sac is opened and the fetus is carefully transferred to a new sterile microscope slide. The crown-to-rump length (CRL) of the fetus is measured by appositioning the back of the fetus next to a millimeter scale. It is recommended that two to three fetuses in each litter are measured to secure gestational age, as some small individual variation between fetuses can exist (see Seiger, this volume). Using Vannas' iridectomy scissors and sharp Dumont no. 5 watchmaker's forceps the embryonic brain is removed under the dissecting microscope.

It is important to remove the skin, the underlying mesenchyme and the meninges as completely as possible without damaging the brain. Always pull away from the brain to avoid damage. The brain is transferred to glucose-saline medium in a black glass dish. With the fetal stages used here, dissection in liquid gives stability to the otherwise soft and jelly-like embryonic

CNS tissue. Dissections are performed with the small iridectomy scissors, avoiding any tearing of the tissue to be grafted. Care should be taken to remove any pieces of meninges that are attached to the piece dissected. Once free from the rest of the brain the dissected piece ( $1-2\text{ mm}^3$  in size) is floated onto the tip of the scissors or forceps and transferred to a collecting dish containing 2-5 ml basic saline-glucose medium at room temperature. In general pieces from all embryos of one (or sometimes two) litters (8-15 fetuses per litter) are collected over a period of 30-75 min.

### *II.3. Suspension preparation*

The tissue pieces are carefully transferred to a sterile glass micro test tube (0.4 ml capacity) containing approximately 0.2 ml of the 0.1 % trypsin solution. They are incubated at  $37^\circ\text{C}$  for about 20 min in a small test-tube incubator (this time includes the time taken to reach  $37^\circ\text{C}$ ). The trypsin solution is then withdrawn and the tissue pieces are washed four to six times with the basic medium using a sterile syringe. Finally, basic saline-glucose medium is added to a volume equivalent to about  $10\text{ }\mu\text{l}$  per dissected tissue piece. The tissue pieces are then dissociated into a suspension of mainly single cells, with occasional cell aggregates, by repeated triturating with a Pasteur pipette with a fire-polished opening (diameter about 1 mm). The number of strokes with the pipette necessary to obtain a good and fairly homogenous cell suspension can vary greatly depending on the age of the donor and the region dissected. However, we try to limit pipetting to 20-30 not too forceful strokes in order to minimize cell damage. Take care not to introduce air bubbles. The suspension can readily be used over at least 3-4 h after preparation (see Brundin et al., 1985a, for discussion on cell viability over time in neuronal cell suspensions).

### *II.4. Suspension injection*

Injections are made with a  $10\text{ }\mu\text{l}$  Hamilton syringe (mounted in a stereotaxic frame) with sterile 23-27-gauge needles with the tips ground down to a  $45^\circ$  angle. Suspension is drawn up into the syringe when it is mounted in the frame. It is very important to ensure repeatedly during the operating session that there is free flow of suspension through the needle as it can easily become blocked. With time the cells in the suspension will sediment to the bottom of the micro test tube and partially reaggregate. Gentle tapping on the test tube may be sufficient to return the cells to suspension, otherwise a few gentle strokes with the Pasteur pipette can be performed to reagitate the suspension prior to withdrawing a new aliquot.

The graft recipients are anesthetized, for example, by a short-acting barbiturate (methohexital; Brietal, Lilly, 40 mg/kg) and placed in the stereotaxic frame. The injection needle is lowered to the predetermined coordinates through a small burr hole in the cranium. One to 3  $\mu$ l of suspension are deposited at a rate of 1  $\mu$ l per minute at each site and the needle is left in situ for an additional 2-5 min. Occasionally, the cell suspension is found on microscopic analysis to have in part flowed back along the needle tract. This reflux can probably be minimized by using small injection volumes and leaving the needle in situ for an adequate time period. At least two deposits can be made along the same needle tract, and several needle penetrations can be made during the same operative session (see Björklund et al., 1983b, for further discussions on this point). We do not use any immunosuppressive treatment or antibiotics in conjunction with the surgery.

## II. 5. Modifications

Some alternatives to the dissociation procedure presented above have been reported. Schmidt et al. (1983) used Earles basic salt solution (EBSS), instead of the saline-glucose medium, and included 0.0125% soybean trypsin inhibitor (Sigma type 1-S), 0.004% DNAase (Sigma type I) and  $\text{MgSO}_4$  (15 mM) in the washing and dissociation step. DNAase, in particular, was used in order to prevent reaggregation of cells due to release of DNA from damaged cells. Floeter and Jones (1984) have reported successful results with suspensions prepared in a medium free of divalent cations in grafting to microcephalic neonatal rats, and Freed et al. (this volume) have used purely mechanical dissociation, through a 250  $\mu$ m monofilament mesh, for dissociation of adrenomedullary cells used for injections into the striatum of adult rats. In our own recent experiments (Brundin et al., 1984), finally, we have obtained good results with mesencephalic dopamine neurons dissociated in a glucose-saline medium containing EDTA instead of trypsin.

## III. Cell Counts and In Vitro Viability

To assess the viability of the cell suspensions used for grafting we have employed a vital stain consisting of acridine orange (Sigma) and ethidium bromide (Sigma) (3.4  $\mu$ g/ml of each component dissolved in 0.9% saline). A 0.5-2  $\mu$ l aliquot of cell suspension is taken, usually from the same syringe as used for grafting, and mixed with 10  $\mu$ l of the vital stain on a microscope slide. The stained suspension is transferred to a hemocytometer, and is immediately counted under epi-illumination in a fluorescence microscope with simultaneous trans-illumination of white light to visualize the counting

chamber grid. Viable cells only take up acridine orange, which stains their nuclei green, whereas cells with leaky membranes allow ethidium bromide to enter, giving them an orange appearance in the fluorescence microscope (Mishell and Shiigi, 1980). It has been shown that intact membrane properties assessed as above are of importance for their subsequent survival after grafting, and that suspensions with less than 50% of the cells viable give generally poor results (Brundin et al., 1985a). We recommend, therefore, that viability assessments are conducted directly after preparation of the suspension and again some hours later, when it is possible that the viability has declined. Cell counting also makes it possible to standardize and balance the injected cell numbers between animals and operating sessions by adjusting the volume of suspension injected. Our experiments with mesencephalic dopamine cells (Brundin et al., 1985a) have indicated that the minimum number of cells to be injected for reproducible good results in the 6-hydroxydopamine-denervated striatum is of the order of 100,000 (the ratio between surviving dopamine cells in the graft to the total number of cells injected varied between 1:100 and 1:1000).

## **IV. Comments on the Procedure**

### *IV.1. Age of donor*

The developmental stage of the tissue is critical for good neuronal survival in the suspension grafts (Björklund et al., 1980; Schmidt et al., 1981). Table I summarizes the fetal donor ages for different CNS regions which have yielded good results in experiments conducted so far. In the case of mesencephalic dopamine neurons we know that good survivals are obtained with donors up to about day 15 of gestation. Donors a mere 12–36 h older gave drastically reduced cell survival. This difference is also partly reflected in the *in vitro* viability counts (Brundin et al., 1985a). Similarly, cerebellar Purkinje cells have been observed to survive in large numbers from ED15 donors, but not from ED18 or older. By contrast, cerebellar microneurons (which continue to be generated up into the postnatal period) survive also when taken from late embryonic or early postnatal stages. These observations suggest that CNS neurons survive dissociation and grafting well only when taken during their period of proliferation and migration.

### *IV.2. Dissociation parameters*

No direct comparison has so far been conducted between different alternative procedures currently in use. Thus, the importance of various parameters

in the incubation and dissociation steps, or the value of more complex dissociation media, is unclear. With the simple standard procedure we use, we have observed good survival from a wide variety of neuronal cell types (see Table I). However, we have recently observed that the noradrenergic neurons of the fetal locus coeruleus are sensitive to trypsin, and that these neurons will not survive suspension grafting unless the trypsin incubation step is omitted (Björklund et al., 1983a). Thus, for certain neuron types dissociation without trypsinization may be necessary.

### *IV.3. Injection parameters*

Although multiple injections are feasible, it is likely that the total volume injected during one session has to be kept within certain limits. In one experiment (Björklund et al., 1983b) we injected as much as 20  $\mu$ l, spread over five injection sites, during 30 min, but the overall survival was poor. Volumes of this magnitude probably increase the intracranial pressure, resulting in increased cell loss through back-flow. The use of smaller volumes and more concentrated cell suspensions should, however, help to overcome this problem.

## **V. Pros and Cons of the Technique**

*The limitations* of the suspension grafting technique are primarily related to the fact that the implanted grafts often are more difficult to delineate and that they become less well organized internally. Tracer injections or electrical recordings within the graft will therefore meet with greater difficulties than with, for example, solid grafts in superficial transplantation cavities. This problem can, however, be partly overcome when the suspension grafts are included in slice preparations, such as in the hippocampal slice studies reported by Segal et al. in this volume. Migration of cells from the suspension grafts is known to occur, although the magnitude of this phenomenon may vary considerably depending on cell type, developmental stage, etc. Proper identification of the graft cells under these conditions will require specific cellular markers (see the chapters by Gumpel et al., Jones and Floeter, Lindsay and Raisman, and Charlton et al. in this volume for examples of such markers).

*The advantages* of the technique can be summarized in the following points.

- (1) The procedure allows grafting to any predetermined site in the brain or spinal cord. It is relatively non-traumatic and can be used in intact brains

(e.g. in experiments on normal development and aging) when one wants to keep the damage to the host to a minimum.

(2) The technique allows multiple grafting simultaneously in several different sites from the same cell suspension. This makes possible, for example, reinnervation of larger areas in the previously denervated adult brain or spinal cord.

(3) Cell counting and viability assessment prior to grafting should increase the possibilities to make reproducible grafts in large series of experimental animals.

(4) Suspension grafts have interesting potentials with respect to manipulation, purification or mixing of cells prior to grafting. Cell sorting techniques, currently tested in in vitro systems with encouraging results, should thus be possible to use as a means of obtaining more pure populations of individual cell types. Cell cultures and in vitro established cell lines, which have been shown to survive re-dissociation and re-plating (Denis-Donini et al., 1983), should be possible to use as well. Finally, ongoing experiments (Brundin et al., 1985b) have shown that the grafting of mixtures of cells from different anatomical regions (a neuronal population and its target region) is feasible. This may provide new opportunities to study, for example, factors influencing cell survival and fiber outgrowth.

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